Reprod Nutr Dev (1997) 37, 41-49

Original article

Comparative analysis of in vitro development of outbred mouse embryos cultured in Krebs-Ringer or tyrode-derived media

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(Received 19 August 1996; accepted 13 September 1996)

Summary — Culture media for mouse preimplantation development are usually derived from two basic solutions: Krebs-Ringer and Tyrode. We have used outbred mice (OF1) to make a comparative analysis of derived media of both types and their success in sustaining development from the one-cell to the blastocyst stage. The best results (up to 50%), in terms of overcoming the two-cell block and sustaining development to expanded blastocyst, were obtained with media derived from Tyrode's solution and with Krebs-Ringer-based media in which the high concentrations of certain metabolites (Cl^-, K^+, Mg^{2+} and PO_4^{3+}) were reduced to the values present in the Tyrode-derived media. Interestingly, medium T6 (based on Tyrode and incapable of avoiding developmental block on its own), yielded the best development rate with the addition of ethylenediaminetetraacetic acid (EDTA). The addition of glutamine to medium T6 did not have any effect. We further developed T7, a medium based on T6 but incorporating the concentrations of nutrients present in the oviduct. This medium also proved better than its Krebs-Ringer counterpart (MTF), but not better than T6, probably due to the different nutritive requirements in the transition from morula to blastocyst. The two most significant findings were: i) that Tyrode-based formulations were superior to media based on Krebs-Ringer solution, and ii) that medium T6 with EDTA was as effective for mouse egg culture as the recently developed KSOM medium.

culture media / embryo / outbred mice / preimplantation stages

Résumé — Analyse comparative du développement in vitro d'embryons de souris non consanguines cultivés dans des milieux à base de Krebs-Ringer ou de Tyrode. Les milieux de culture adaptés au développement préimplantatoire de la souris sont habituellement dérivés

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de deux solutions de base : Krebs-Ringer et Tyrode. Nous avons utilisé des souris non consanguines (OFI) pour effectuer une analyse comparative de milieux dérivés des deux types et de leur efficacité pour assurer le développement du stade une cellule au stade blastocyste. Les meilleurs résultats (jusqu'à 50 %), en ce qui concerne le dépassement du bloquage au stade deux cellules et le maintien du développement jusqu'au stade blastocyste, ont été obtenus avec des milieux dérivés de la solution de Tyrode et avec des milieux dérivés du Krebs-Ringer dans lesquels les fortes concentrations de métabolites (Cl^-, K^+, Mg^{2+} et PO_4^{3+}) ont été réduites aux valeurs présentes dans les milieux dérivés du Tyrode. Remarquablement, le milieu T6 (basé sur le Tyrode et incapable d'éviter le bloquage de lui-même) permet le meilleur taux de développement après addition d'EDTA. La glutamine ajoutée au milieu T6 n'a aucun effet. Nous avons ensuite développé le milieu T7, basé sur T6 mais incorporant les concentrations de nutriments présents dans l'oviducte. Ce milieu s'est avéré meilleur que sa contrepartie basée sur le Krebs-Ringer (MTF), bien qu'il ne soit pas meilleur que T6, probablement à cause du changement d'exigences nutritives lors de la transition morula-blastocyste. Les deux résultats importants sont : i) les formules basées sur le Tyrode sont supérieures à celles basées sur le Krebs-Ringer ; ii) le milieu T6 avec EDTA est aussi efficace pour la culture de l'œuf de souris que le milieu KSOM développé récemment.

milieux de culture / embryon / développement préimplantatoire / souris non consanguines

INTRODUCTION

In vitro development of preimplantation mouse embryos is far from optimum as can be inferred by the 18–24 h delay in cleavage in in vitro cultures compared with in vivo rates, and by the arrest shown by most strains at the two-cell stage when cultured from the one-cell stage, a phenomenon commonly known as the 'in vitro two-cell block'. Only the embryos of certain inbred strains, and their F_1 hybrids, develop consistently from the one-cell to the blastocyst stage under these conditions (Harlow and Quinn, 1982; Pratt, 1987). It has been shown that this block in cell division is a phenomenon regulated by cellular factors of maternal origin that may be solved when the cytoplasm from a non-blocking embryo is injected into a blocking one (Muggleton-Harris et al, 1982). It is not known what the cytoplasmic factor confers to the non-blocking strain's ability to survive under conditions outside the maternal environment.

There are several methods, such as the co-culture of embryos with oviduct and uterine cells (Sakkas and Trounson, 1990), that have been used to improve culture media thus facilitating the in vitro development of outbred mice. Similarly, the addition to culture media of micromolar concentrations of ethylenediaminetetraacetic acid (EDTA) (Abramczuk et al, 1977) and glutamine, the removal of glucose from media and the variation of the lactate/pyruvate ratio and the concentration of different salts have improved in vitro development of one-cell embryos from random-bred mice (Chatot et al, 1989; Lawitts and Biggers, 1992; Erbach et al, 1994). All these changes have been made in chemically defined media based on the Krebs-Ringer bicarbonate solution containing lactate, pyruvate, glucose and bovine serum albumin (BSA) (Whitten, 1957; Brinster, 1965a,b,c). A second group of media based on Tyrode's solution and supplemented in the same way has been used to a lesser extent (Harlow and Quinn, 1982; Quinn et al, 1984; Dandekar and Glass, 1987; Sakkas and Trounson, 1990). The principal difference between the two groups is the higher Cl^-, K^+, phosphate and Mg^{2+} concentrations in the Krebs-Ringer-

derived media. These variations could account for the different capacities to sustain culture throughout the preimplantation period, as has been shown by Lawitts and Biggers (1991), who found that high concentrations of NaCl, pyruvate, KH_2PO_4 and glucose are detrimental to embryo development. However, very few comparative analyses have been made to date between these two groups of media and no conclusive results exist. Dandekar and Glass (1987) reported the positive effect of a Tyrode-derived medium (T6) compared with other media of different origin, but only when supplemented with preovulatory human serum. In addition, Spindle (1990) described an important improvement using a Krebs-Ringer-based medium (TE) but with T6 concentrations of certain salts, which in fact meant the development of a new Tyrode-derived medium. Since then, more improvements have been published, mainly in the Krebs-Ringer-derived group of media (Erbach et al, 1994), although comparative analysis with previous results is lacking in the literature. A further problem is the difficulty to compare the published data because of the considerable variation in the success obtained by each medium using different strains of mice in different laboratories (Dandekar and Glass, 1987; Erbach et al, 1994).

The purpose of this study was to determine the effects of different media, based on either Krebs-Ringer (M16, WM and CZB) or Tyrode formulations (TE and T6) on the development of preimplantation embryos from an outbred strain of mice from the one-cell to the blastocyst stage. Tyrode-derived media were also compared with KSOM, a recently developed medium using the simplex optimization method which yields up to 88% of blastocyst development (Erbach et al, 1994). The effects on the development of EDTA, glutamine and more physiological concentrations of nutrients (Gardner and Leese, 1990) were also assayed in a new medium called T7.

MATERIALS AND METHODS

Embryo collection

Random-bred outbred (OF1) female mice (Iffa-Credo, Lyon, France) of 6 weeks of age were superovulated by intraperitoneal injection at midday using 5 IU pregnant mare's serum gonadotropin (PMSG; Intervet, Salamanca, Spain) followed 48 h later by 5 IU human chorionic gonadotropin (hCG; Serono, Madrid, Spain). The females were caged with OF1 males and mating was confirmed the following morning by the presence of vaginal plugs; the day of the vaginal plug was considered day 1 of development. Ovulation and mating were assumed to occur 12 h after hCG administration. One-cell embryos were obtained 19–20 h post-hCG injection from the ampulla of the oviduct in warm MA-1, a T6-derived medium (Howlett et al, 1987) buffered with 20.8 mM HEPES (Boehringer Mannheim, Germany) and a smaller concentration of sodium bicarbonate (4.15 mM; Merck, Darmstadt, Germany). Osmolarity was adjusted to 265 mOsm with NaCl (final concentration 95.7 mM). Eggs were denuded from cumulus cells by a brief incubation with a 0.1% hyaluronidase solution (Sigma, St Louis, MO, USA) (Hogan et al, 1986). Zygotes were then washed three times in MA-1 medium before being placed in culture.

Culture media

The embryos were cultured in six basic media (table I): modified Whittingham medium 16 (M16) (Chatot et al, 1989), modified Whitten's medium (WM) (Hoppe, 1985), CZB medium (Chatot et al, 1989), KSOM from Erbarch et al (1994), TE medium (Spindle, 1990) and modified T6 (Howlett et al, 1987). EDTA (0.02 mM) was added to all media which did not include it in their original formulation (all but CZB and TE). The effect of glutamine (1 mM; Sigma) in Tyrode-derived formulations was assayed in T6 medium. Finally, T7, a medium based on T6 but with the concentrations of nutrients (lactate and pyruvate) replaced by those in the oviduct fluid

Table I. Composition (mM) of culture media for mouse embryos.

	M16	*WM*	*CZB*	*KSOM*	*TE*	*T6*	*T7*
NaCl	94.62	109.51	81.62	95	97.86	80.77	100.25
KCl	4.83	4.83	4.83	2.5	1.42	1.48	1.48
NaH_2PO_4	–	–	–	–	–	0.39	0.39
Na_2HPO_4	–	–	–	–	0.36	–	–
KH_2PO_4	1.18	1.18	1.18	0.35	–	–	–
$MgCl_2$	–	–	–	–	0.47	0.49	0.49
$MgSO_4$	1.18	1.18	1.18	0.2	–	–	–
$CaCl_2$	1.70	–	1.70	1.71	–	1.77	1.77
$NaHCO_3$	25	22.62	25.12	25	22.52	25	25
Na lactate	22	–	31.30	10	21.55	23.29	4.79
Ca lactate	–	4.86	–	–	1.76	–	–
Na pyruvate	0.55	0.27	0.27	0.2	0.25	0.27	0.37
D-glucose	5.55	5.55	–	0.2	5.55	5.55	0.55
Glutamine	–	–	1	1	–	–	–
Penicillin G*	62.8	62.8	62.8	62.8	62.8	62.8	62.8
Streptomycin*	50	50	50	50	50	50	50
Phenol red*	–	–	–	0.01	0.001	0.01	0.01
BSA*	4	3	5	1	3	4	4
EDTA	0.02	0.02	0.11	0.01	0.02	0.02	0.02

*Penicillin G, streptomycin, phenol red and bovine serum albumin (BSA) are in mg/mL.

according to Gardner and Leese (1990), was tested. Osmolarity of T7 was adjusted to 267 mOsm with NaCl. Osmolarity of the rest of the media ranged from 270 to 280 mOsm, which are within the optimum range of osmolarities determined by Brinster (1965b).

The media were prepared from stock solutions x 100 with the following exceptions: NaCl, KCl, NaH_2PO_4, $MgCl_2$ and phenol red were stored together x 10. $NaHCO_3$ was kept x 40, glutamine x 200 and EDTA x 500. All chemicals were purchased from Sigma (cell culture grade and embryo tested if possible) and Merck. Solutions were stored at –20 °C in cryogenic vials (Corning, NY, USA) for up to 2 months except $NaHCO_3$, sodium pyruvate and glutamine, which were made every 2 weeks. Culture media were freshly prepared every 15 days using embryo-tested water (Sigma). Glutamine, EDTA and BSA (fraction V, Sigma) were added at the moment of media reconstitution. Finally, media were filtered into 15 mL polypropylene tubes (Corning) with a 0.2 µm cellulose acetate filter unit (Lida, WI, USA).

Culture of embryos and statistical analysis

The embryos were cultured in groups of 20 in 12 µL droplets of medium under equilibrated mineral oil (Sigma) in 35 mm petri dishes (no 1008, Falcon, Plymouth, UK) (Brinster, 1963; Wiley et al, 1986). They were kept for 5 days in a 5% CO_2 incubator at 37 °C. The percentage of embryos reaching the two- and four-cell stages after 24 and 48 h, and the compacted morula and expanded blastocyst stages after 72 and 120 h of culture, respectively, were determined under a Leitz M10 microscope x 80.

Statistical analysis of the data was carried out by analysis of variance (ANOVA) and Student's *t*-test with the aid of the SPSS/PC+ sta-

Table II. Development of one-cell mouse embryos cultured for 5 days in different media.

	M16 (n = *124*)	*WM* (n = *117*)	*CZB* (n = *121*)	*TE* (n = *123*)	*T6* (n = *122*)	*P*
Two-cells	88	89.1	86.68	90.3	88.2	0.9711
Four-cells	0.3	9.4	45.75*	44.1*	58.7*	0.0001
Morula	0	3.3	45.62*	51.5*	47*	0.00001
Blastocyst	0	10.2	40.32*	37.7*	50.7*	0.00001

Results (in %) of five replicates. *Statistically significantly different from M16 and WM, by Duncan's test, $P \leq 0.05$.

tistical package (Microsoft Co, Seattle, WA, USA). Duncan's test for multiple comparisons between paired means was further applied to detect significant ($P \leq 0.05$) differences between means.

RESULTS

The data on the stage of development reached after 24, 48, 72 and 120 h in culture for each of the basic media tested are shown in table II. On day 2 of culture the percentage of two-cell embryos was similar in all groups. However, embryos maintained in M16 and WM significantly arrested at this stage regardless of the 0.02 mM EDTA added. Only embryos cultured in CZB and in the Tyrode-derived formulations (TE and T6) overcame this block, although only partially, with a percentage of embryos reaching the four-cell stage that ranged from 45 to 60%. Finally, more than 85% of these four-cell embryos reached the expanded blastocyst stage after another 3 days of culture. Both TE and CZB media yielded similar results, allowing the development to the blastocyst stage of 40 and 38% of the embryos, respectively. However, the best capacity for sustaining embryo development was obtained with T6 medium, which was the most successful medium throughout the culture period, especially in the transition from morula to blastula. In fact, with this medium most morula and even some eight-cell embryos between the third and fifth day of culture reach the expanded blastocyst stage. In total, up to 50% of embryos developed to blastocyst, which is an important percentage considering that the original formulation of this medium (without EDTA) is incapable of over-

Table III. Development of one-cell mouse embryos cultured in T6 and KSOM media.

	Two-cells	*Four-cells*	*Morula*	*Blastocyst*
T6 (n = 219)	76.1	57.2	59.1	45.6
KSOM (n = 222)	73.7	53.1	51.9	45.5

Results (in %) of four replicates.

coming the two-cell block (personal observation).

We then assayed the effect of enriching T6 with 1 mM glutamine. However, there was no difference between this variety and T6 lacking the amino acid. The percentage of embryos reaching the different stages tested was similar in all cases (not shown).

Having established that T6 medium was the best formulation, we compared it with KSOM (table III), a recently developed medium yielding the best results described to date for blastocyst development with OF1 mice (88%). We were able to confirm that KSOM clearly represents an improvement over the development obtained with other media based on Krebs-Ringer, although in our experiments with OF1 mice the results were not as high as those reported in the literature (Erbach et al, 1994). In fact, the percentage of embryos reaching the blastocyst stage was similar to the rate obtained with T6.

Finally, we tried to improve T6 medium by replacing its nutrients (lactate and pyruvate) with those concentrations present in the maternal oviduct fluid. Table IV shows the ability of this medium (called T7) to sustain development as compared with T6. It is clear that there are no differences between the two from the one-cell stage to morula. In fact, the development of embryos cultured in T7 was slightly poorer than in T6. Interestingly, this tendency was more apparent after the morula stage when T7 appeared to be detrimental to blastocyst growth.

DISCUSSION

In the present study a comparative analysis was made between culture media derived from Krebs-Ringer and Tyrode's solutions. The ability of different media to support development is directly related to the capacity of embryos to avoid blocking at the two-cell stage. The most frequent methods used in solving this problem have been to add EDTA and/or remove glucose from the culture media (Abramczuk et al, 1977; Mehta and Kiessling, 1990; Scott and Whittingham, 1996). Three of the media we assayed did not include EDTA in their original formulations (M16, WM and T6) and were therefore unable to overcome the two-cell block. However, their responses after adding 0.02 mM EDTA were completely different. While neither M16 nor WM could sustain significant embryo development, T6 proved to be the most effective culture media of all those tested with OF1 embryos. In fact, it was better, although not significantly, than CZB, a medium specifically developed to avoid the problem of blocking in strains of mice (Chatot et al, 1989). The main differences between CZB and previous formulations were the absence of glucose for the first 48 h of culture, the presence of glutamine and EDTA and an increased lactate/pyruvate ratio of 116. However, the success of CZB cannot be attributed solely to these variations since Tyrode-derived formulations (TE and T6) contain glucose from the beginning, their lactate/pyruvate ratio is much smaller (around 90), the addition of glutamine does not have any significant effect and EDTA

Table IV. Development of mouse embryos cultured in T6 and T7 media.

	Two-cells	*Four-cells*	*Morula*	*Blastocyst*
T7 (n = 115)	77.4	52.1	43.5	39
T6 (n = 122)	79	54.8	48.5	47.7

Results (in %) of four replicates.

by itself may not be completely effective, as can be appreciated in other Krebs-Ringer-derived media (M16 and WM). Thus, the excellent results obtained with T6 compared with classical blocking media such as M16 and WM must be accounted for by other means.

Recently a new culture medium called KSOM has proved to be even better than CZB (Erbach et al, 1994). In fact, the authors obtained 88% development to the blastocyst stage with this medium, compared to 52% with CZB. In our experiments, KSOM was also shown to be the best Krebs-Ringer-derived medium, but surprisingly it was not better than T6 (table III). The reason for this may be provided by several authors (Dandekar and Glass, 1987; Spindle, 1990; Scott and Whittingham, 1996) who reported that different random-bred strains of mice have distinct responses when cultured in the same medium. Thus, the high percentage of embryo development found by Erbach et al (1994) might be specific to the strain of mice used, which resulted from mating CF1 females with hybrid BDF males. However, regardless of strain-specific effects, the most interesting result of our comparative analysis is that none of the improvements in the Krebs-Ringer-based media produced a medium better than Tyrode-derived T6.

The principal differences between media derived from Krebs-Ringer solution and those from Tyrode (TE and T6) are the concentrations of certain metabolites such as Cl^-, K^+, Mg^{2+}, phosphate (PO_4^{3+}) and glucose in the case of the CZB and KSOM, which are smaller in T6 and TE. Table V shows that a direct relationship can be established between high Cl^- and K^+ concentrations and blockage at the two-cell stage. In fact, only those Krebs-Ringer-derived media that include glutamine in their formulations proved capable of overcoming the block. It is not known how glutamine confers this advantage to these media, but it is most likely used to protect the embryo in the presence of high concentrations of sodium chloride (Lawitts and Biggers, 1992; Ali et al, 1993). These high Cl^- concentrations could alter the Cl^-/HCO_3^- antiport present in the two-cell embryo, which would involve a decrease in the pH_i (Lawitts and Biggers, 1991; Baltz, 1993). In this regard, we detected no effect of glutamine in a medium with a low concentration of Cl^- such as T6. In fact, Cl^- and K^+ could act synergistically since relatively high Cl^- concentrations in TE – a medium with a low K^+ concentration and no glutamine – do not seem to be detrimental. In the same way, Wiley et al (1986) observed that 6 mM of potassium was detrimental to embryo development compared with 1.4 mM. Our data seems to confirm the inhibitory effect of raising the concentration of K^+ to 6 mM (table V). Phosphate, in the form of KH_2PO_4 or Na_2HPO_4, is also present in Krebs-Ringer- and Tyrode-derived media. Previous reports indicate that reducing phosphate to a low level increases embryo development both in mice (Lawitts and Biggers, 1991) and hamsters (Schini and Bavister, 1988). Similarly, phosphate seems to mediate the negative effect of high glucose concentrations in the two-cell

Table V. Total concentration (mM) of selected metabolites in culture media.

	M16	*WM*	*CZB*	*KSOM*	*TE*	*T6*	*T7*
Cl^-	103	114.2	90	100.9	100	86.5	106
K^+	6	6	6	2.85	1.42	1.48	1.48
PO_4^{3+}	1.18	1.18	1.18	0.35	0.3	0.3	0.3
Mg^{2+}	1.18	1.18	1.18	0.2	0.47	0.49	0.49
Pyruvate	0.55	0.27	0.27	0.37	0.25	0.25	0.37

block (Scott and Whittingham, 1996). Here again we have seen that the poorest media are those with high PO_4^{3+} concentrations and that high glucose does not seem harmful when low phosphate is used. Similar conclusions can be drawn regarding magnesium. Finally, Lawitts and Biggers (1991) showed that pyruvate levels approaching 1 mM resulted in a decrease in the percentage of developing embryos. In our case this could help to explain the poor results obtained with M16.

It is difficult to attribute to only one of these substances sole responsibility for low embryo development rates. The combined effect of high concentrations of all of them is more likely to be the cause of such results, which can only be ameliorated with glutamine in an unknown form. It is interesting to note that KSOM, the best Krebs-Ringer-derived medium, has relatively low amounts of Cl^- and concentrations of K^+, PO_4^{3+} and Mg^{2+} were more similar to those of T6 and TE than M16, WM and CZB. This medium was developed using a strategy called 'simplex optimization', which is able to optimize several components simultaneously (Lawitts and Biggers, 1991; Erbach et al, 1994). Curiously, the result of this method is a medium that is much closer to the original Tyrode-derived formulations than to the classical culture media based on Krebs-Ringer, which favours the hypothesis of a beneficial effect of low concentrations of certain metabolites.

It is surprising to observe, however, that the concentrations of Cl^- and K^+ considered beneficial for in vitro development are much lower than those found in the mouse oviduct (Lawitts and Biggers, 1991). The concentrations of nutrients, mainly lactate, are also different from those present in culture media (Gardner and Leese, 1990). We have observed that replacing the concentrations of T6 nutrients with those found in the oviduct does not yield a higher rate of blastocysts, and may even produce a lower number, although the two-cell block was avoided. T7 seems to be especially harmful in the transition from the morula to blastocyst stage, when embryos start using glycolysis to generate energy (Barbehenn et al, 1978; Leese and Barton, 1984; Brown and Whittingham, 1991, 1992). In a previous paper, Gardner and Leese (1990) reported similar results with non-blocking strains of mice comparing M16 with MTF, a medium in which the oviductal concentrations of lactate and pyruvate replaced those of M16. However, when outbred embryos were assayed in MTF, they blocked at the two-cell stage (Erbach et al, 1994). Our data with T7 not only confirm the differences between the optimal conditions in vivo and in vitro but also the improvement brought about by decreasing Cl^-, K^+, PO_4^{3+}, Mg^{2+} and pyruvate concentrations, since embryos did not block. We therefore consider that regardless of the different responses that can be observed when using different strains of mice, low concentrations of these metabolites, as are found in the original Tyrode-derived formulations, are essential for developing culture media for preimplanted embryos.

ACKNOWLEDGMENTS

We thank Professor R Brinster for his critical reading of this paper. This study was partially supported by grants from the Spanish Ministry of Education and Science (CICYT PB92.0438), the Basque government (PGV 9203) and the University of the Basque Country (UPV 075.310-EBO14/92) to JA, JAU was a recipient of a fellowship from the Basque government. We are grateful to RG Tobalina for help with the medium KSOM.

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